Selecting and Sorting Waxy Wheat Kernels Using Near-Infrared Spectroscopy

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ABSTRACT

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An automated single kernel near-infrared (NIR) sorting system was used to separate single wheat (*Triticum aestivum* L.) kernels with amylose-free (waxy) starch from reduced-amylose (partial waxy) or wild-type wheat kernels. Waxy kernels of hexaploid wheat are null for the granule-bound starch synthase alleles at all three *Wx* gene loci; partial waxy kernels have at least one null and one functional allele. Wild-type kernels have three functional alleles. Our results demonstrate that automated single kernel NIR technology can be used to select waxy kernels from segregating breeding lines or to purify advanced breeding lines for

the low-amylose kernel trait. Calibrations based on either amylose content or the waxy trait performed similarly. Also, a calibration developed using the amylose content of waxy, partial waxy, and wild-type durum (*T. turgidum* L. var *durum*) wheat enabled adequate sorting for hard red winter and hard red spring wheat with no modifications. Regression coefficients indicated that absorption by starch in the NIR region contributed to the classification models. Single kernel NIR technology offers significant benefits to breeding programs that are developing wheat with amylose-free starches.

Amylose-free hexaploid (*Triticum aestivum* L.) or tetraploid (*T. turgidum* L. var *durum*) wheat is referred to as waxy, which is a term first associated with low-amylose corn (*Zea mays* L.) because the kernels have a waxy appearance. This follows the convention established in other cereal crops (Boyer and Hannah 1994) and occurs when all waxy genes (*Wx* loci) controlling synthesis of granule bound starch synthase (GBSS) are null. In the polyploid wheat species, partial waxy genotypes are classified as having at least one null and one wild-type *Wx* gene. Wild-type kernels have no null *Wx* genes and all functional GBSS alleles.

Amylose-free wheat has functional end-use advantages over amylose-bearing wheat, and these advantages can provide additional marketing opportunities. For example, low-amylose wheat flour has a high water-binding capacity; products made from waxy flour can exhibit a longer shelf-life; products extruded from waxy flour can be enhanced; and ethanol conversion from low-amylose wheat is enhanced (Graybosch 1998; Chibbar and Chakraborty 2005; Hung et al 2006; Wu et al 2006).

Several wheat breeding programs are developing waxy genotypes in an attempt to take advantage of potential new markets and capitalize on the unique functionality of waxy flour. The first waxy wheats were developed by Nakamura et al (1995). However, after crosses are made between waxy and wild-type parents, as few as 1 in 64 progeny may express the waxy trait. Therefore, the ability to select waxy seed from early-generation segregating populations would provide breeding materials enriched in the number of seeds with this trait. Also, if an advanced waxy breeding line requires purification for the waxy trait before release, there is no existing efficient or cost-effective way to accomplish this, especially because large seed samples are typically involved.

Waxy seed can be identified using SDS-PAGE (Zhao and Sharp 1996), enzyme-linked immunosorbent assay (ELISA) (Rahman et al 1995), genetic markers (Nakamura et al 2002), or by iodine staining (Pedersen et al 2004). However, these methods are not condu-

cive to identifying and selecting large numbers of seeds and may be cost-prohibitive. Delwiche et al (2006) analyzed single seeds of durum wheat with near-infrared spectroscopy (NIRS), and could discriminate between waxy kernels and partial-waxy or wild-type kernels using this fast and nondestructive procedure. They could automatically scan plates of 48 kernels but the instrument could not automatically position kernels on the plate or automatically sort them after analysis. However, automated NIR sorting could be used to enrich early-generation populations and provide breeders with access to such populations from which waxy lines could be selected. Dowell et al (2006) developed an automated single kernel NIR (SKNIR) system that could automatically scan and sort single proso millet (*Panicum miliaceum* L.) seeds into waxy and nonwaxy fractions, but this technology has not been applied to sorting waxy wheat. The objective of this research was to determine whether automated SKNIR technology could separate waxy from nonwaxy wheat kernels and validate the calibration using kernels from multiple crop years and two wheat classes.

MATERIALS AND METHODS

Sample Description

Samples of durum, hard red spring (HRS), and hard red winter (HRW) wheat were obtained from 2003-2007 crop years (CY) (Table I). For all tests, samples were sorted to return approximately pure waxy seed to the respective breeding program.

Durum samples. Delwiche et al (2006) provide details on the durum samples. Briefly, 60 durum wheat lines were obtained from five populations by selecting three lines having each of four possible GBSS genotypes. These 60 lines were planted at Yuma, Arizona, and 53 F5 lines were harvested in the spring of 2003. Of the harvested lines, only 40 produced enough seed so that samples could be sorted to return >100 g of seed that was needed for subsequent testing. The 53 lines were planted in three replicates in 2003 and the F6 lines were harvested in the spring of 2004. One entire replicate and 23 samples from the other two replicates were not of sufficient size for subsequent testing, leaving 83 samples.

HRW samples. Three replicates of 50 HRW F3 lines were harvested in 2005, but only 117 had sufficient seed quantity or genotypic data for subsequent testing. The 117 samples had an average initial sample size of 353 g and an average of 36% waxy kernels. Forty grams of waxy seed was needed for breeding purposes.

The low-amylose content seeds from the 2005 amylose-content sorts were planted to yield an F4 set of 50 samples harvested in Fall 2007 (hereafter referred to as 2007a samples). Only those harvested samples with a sufficient sample size and a high waxy percentage were selected for sorting. Thus, 14 samples of ≈500 g were selected from 2007a samples.

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Class	Crop Year	No. Samples	Calibration Model	Initial Amylose Content or Waxy Kernel Frequency	Final Waxy Kernel Frequency, %
Durum	2003	40	Amylose content	18.7% amylose	97.8
	2004	83	Amylose content	18.9% amylose	97.9
Hard red winter	2005	117	Amylose content	36.4% waxy kernels	92.5
	2007a	14	Waxy	71.0% waxy kernels	91.8
	2007b	28	Amylose content	71.2% waxy kernels	97.6
	2007b	28	Waxy	71.2% waxy kernels	96.7
Hard red spring	2006	51	Amylose content	98.4% waxy kernels	99.5

An additional 28 F4 samples (1 kg) were also sorted. These samples, harvested in 2007 (hereafter referred to as 2007b samples), were derived from the unsorted 2005 F3 populations and thus had not been sorted prior to planting.

HRS samples. A 54-kg sample of a waxy experimental HRS wheat breeding line, NDSW0481, with ≈2% wild-type seed that was developed at North Dakota State University was also sorted to attempt to return to the breeder a pure waxy sample. This line was produced in Cass County, North Dakota, in crop year 2006. Because the SKNIR sorter can only process ≈1 kg/day, this sample was divided into 51 subsamples of ≈1 kg that were sorted on two SKNIR instruments.

Single Kernel Sorting

We used a SKNIR system to collect spectra and to separate waxy from nonwaxy seeds. This instrument was developed by the USDA-ARS Engineering Research Unit and commercialized by Perten Instruments (Stockholm, Sweden). The system can sort single seeds based on traits such as protein content, hardness, and scab damage at a rate of ≈1 kernel/3 sec, or ≈1 kg/day. The system automatically feeds single seeds to a viewing area where spectra (900–1,700 nm) are collected. Kernels are then automatically sorted based on a value predicted from an averaged spectrum. The system is further described by Dowell et al (2006).

Kernels from each sample were sorted into one of four bins using a criteria based on findings reported by Delwiche et al (2006). They showed that durum wheat with one or two functional alleles generally has >20% amylose content and that waxy seeds generally have <3% amylose content. Thus, we set our amylose content calibration sorting criteria to place seeds with a predicted amylose content of ≤11.5% into bins "1" and "2" and thus these bins should have higher proportion of waxy kernels. Bins "3" and "4" contained kernels with a predicted amylose content of >11.5%.

Similarly, when using the waxy trait calibration to select waxy wheat, sorting criteria were set to collect a higher proportion of waxy kernels into bins "1" and "2", while those with a higher proportion of wild-type kernels were directed to bins "3" and "4."

The repeatability of this sorting procedure when using the amylose content calibration was tested by sorting three replicates from each of three randomly picked samples from the 2003 CY and determining the number of waxy and nonwaxy kernels.

Sorting on each sample, except the 2006 sample, was discontinued after the combined weight of bins 1 and 2 reached ≈40 g, which was the minimum amount needed for subsequent planting and development of waxy lines. For the 2006 samples, all 54 samples (1 kg) were sorted to return the maximum amount of waxy seed to the breeding program

Model Development

GRAMS PLSPlus/IQ (Thermo Galactic, Salem, NH) was used to perform a PLS analysis on all data. All spectra were mean centered before analysis. The accuracy of the classification models was determined using weighted correct classification, correlation coefficient (r), coefficient of determination (r^2), and by the standard error of cross validation (SECV) using a one-sample-out procedure (Williams 2001). Over 3 million kernels from crop years 2003 through 2007 were sorted with this technique.

Amylose content calibration. Models were developed to separate kernels by either predicting their amylose content or by predicting their waxy trait. To develop amylose content calibration, 100 seeds from each of the 2003 durum samples were scanned and the spectra were averaged into one spectrum that represented that sample with a known amylose content. A partial least squares (PLS) calibration (4 factors) was used to predict the amylose content of the 2004 durum samples. An additional calibration (four factors) was developed using the combined 2003 and 2004 data and used to predict the amylose content of the 2005 HRW samples and the 2007a HRW samples. A iodine-binding blue complex colorimetry procedure was used for measuring the amylose content (Knutson and Grove 1994). The procedure was modified slightly by Delwiche et al (2006) to enhance precision of diluting the sample for complex formation. The combined amylose calibration was used to sort the 2003, 2004, 2005, 2006, and 2007b samples.

Waxy calibration. For the waxy seed calibration, we developed a calibration to predict the presence or absence of amylose. We selected two samples that had a high percentage of wild-type kernels, and two that were mostly waxy. These four samples were first sorted using the amylose calibration described above to remove any remaining waxy kernels from the wild-type samples, and any wild-type kernels from the waxy samples. The waxy calibration (five PLS factors) was then developed by scanning 65 kernels from the waxy samples and 59 kernels from the wild-type samples and assigning a value of "1" to the kernels from the waxy samples, and a value of "2" to the kernels from the wild-type sample. The 2007a samples were then sorted using this calibration. We also sorted the 2007b samples using the amylose calibration to compare the effectiveness of that calibration to the waxy calibration. The number of waxy and nonwaxy kernels was determined on 48 kernels from each bin by iodine staining (Pedersen et al 2004).

RESULTS AND DISCUSSION

Sorting by Amylose Content

The cross-validation results of the 2003 durum samples showed that the amylose content could be accurately predicted with $r^2 = 0.90$ and SECV = 3.2% (four PLS factors). Similarly, a cross-validation of the 2004 samples showed that the 2004 amylose content was accurately predicted with $r^2 = 0.91$ and SECV = 2.9% (four PLS factors). When the 2003 and 2004 data were combined, the amylose content was predicted with $r^2 = 0.90$ and SECV = 3.2% using four factors in a PLS regression cross-validation. There was little overlap between waxy kernels (those predicted as having <11.5% amylose content) and all other kernels (Fig. 1). When the 2003 calibration was used to predict the 2004 data, results similar to the cross-validation were achieved ($r^2 = 0.90$; STD = 7.9%).

A plot of regression coefficients (Fig. 2) showed distinct peaks at 985, 1130, 1210, 1310, and 1432 nm. These peaks, whether
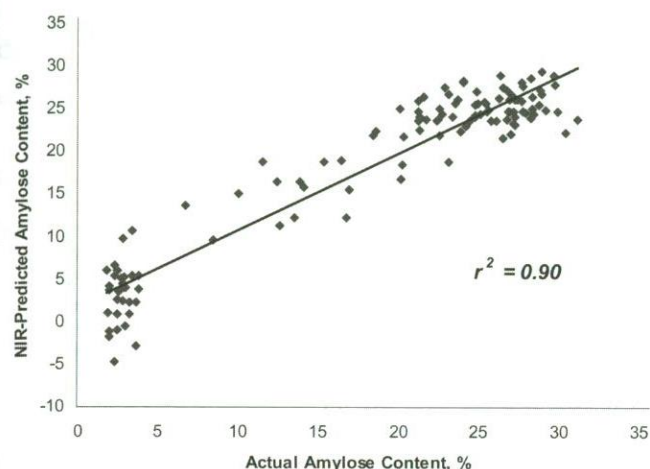
Fig. 1. Actual vs. predicted amylose content when using all samples from 2003 and 2004 crop years and four factors in a partial least squares regression.
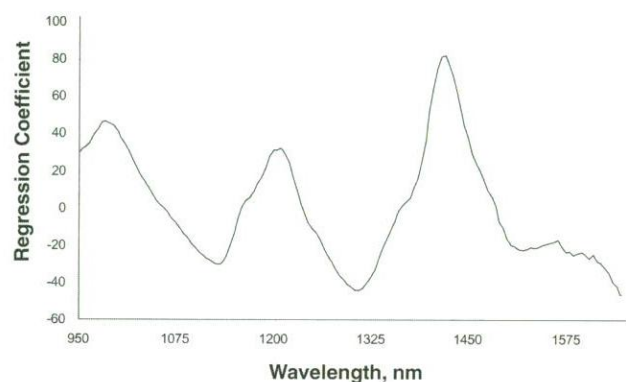

Fig. 2. Regression coefficients of an amylose content calibration model (four PLS factors) developed to select waxy kernels using 2003 and 2004 CY wheat samples. Peaks at 980 and 1,430 nm correspond to starch absorption regions.

Fig. 3. First derivative of absorption spectra showing difference between waxy and wild-type kernels.

Sample Description	Actual		Predicted	
	Average	Std Error	Average	Std Error
Wild-type	27.7a	0.32	24.7a	0.41
Wx-A1 null	22.4b	0.96	21.5b	0.94
Wx-B1 null	21.4b	0.60	22.3b	0.52
Waxy	4.6c	0.79	6.9c	0.93

[a] Values in the same column followed by the same letter are not significantly different ($P < 0.05$).

positive or negative, indicate the absorption regions that are most important in the classification model. The peak at 1430 nm corresponds to a starch principal absorbance band, and the 985 and 1210 nm peaks correspond to weaker starch absorption bands (Williams 2001). The peak at 1430 nm also shows up in the first derivative of the NIR absorbance spectra for averages of waxy and wild-type kernels (Fig. 3). Delwiche et al (2006) identified important wavelengths at 1002, 1140, and 1440 nm, which agree with the regression coefficient peaks shown in this work. Because amylose is a component of starch, the PLS analysis of the NIR spectra is detecting a change in the spectra caused by the presence or absence of amylose.

Table II shows that the average predicted amylose content of waxy kernels was significantly less than nonwaxy kernels. The predicted partial waxy and wild-type amylose contents were also significantly different ($P < 0.05$), but the wx-A1 partial waxy kernels could not be differentiated from partial waxy wx-B1 kernels. This agrees with results reported by Delwiche et al (2006), where the accuracy of correctly classifying partial waxy kernels as wx-A1 null or wx-B1 null was only 11–54%. Their accuracy for classifying kernels as waxy was >90% for most models. Furthermore, Delwiche et al (2006) showed that waxy kernels could be discriminated from partial waxy or wild-type kernels with high accuracy. Because our SKNIR system could also differentiate waxy from partial or nonwaxy kernels with high accuracy, the rest of our analyses concentrated only on differentiating waxy from non-
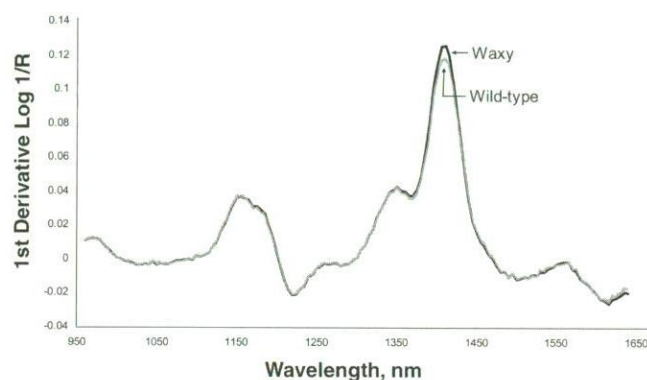
waxy kernels. The amylose content calibration developed from the 2003 and 2004 durum samples was used to sort the 117 2005 HRW samples until 40 g was obtained from the original 350 g sample. The average waxy seed frequency of the unsorted samples was 36.4%, with a range of 3.5–95.7%. After sorting, the final waxy seed frequency in the sample was increased to an average of 92.5% and with a range of 53.7–100% (Table I). Thus, the amylose content durum calibration enabled the sorting of independent HRW samples.

Sorting by Waxy Trait

Because breeding programs are often concerned with obtaining waxy seed from segregating populations, or purifying waxy breeding lines, we examined the feasibility of using the SKNIR sorting technology for selecting waxy kernels using a calibration based on the waxy trait, instead of amylose content. For the 14 samples sorted with this waxy calibration, the average waxy kernel frequency was increased from 71.0% (range 18.8–87.5%) in the unsorted sample to 91.8% (range 75.0–97.9%) in the sorted sample (Table III).

The purity of the sorted portion and the amount of seed discarded can be changed by adjusting the rejection criteria. For example, Table III shows that an average purity of 91.8% can be achieved, but this is at the expense of discarding ≈75% of each sample. However, if the sorting criterion is changed so that only 25% of the sample is discarded then the waxy purity decreases to 87.7% (data not shown).

The waxy calibration used for this sort included five PLS factors and had regression coefficients with some peaks in common with the amylose content calibration (Fig. 2 vs. Fig. 4). The large starch peak at ≈1430 nm is common to the waxy and amylose content regression models.

For the 2007b samples, the waxy seed frequency was increased from 71.2% (range 45.8–85.0%) in the unsorted sample to an average of 96.7% (range 87.5–100%) in the sorted sample (Table I) when using the waxy calibration. About 300 g was sorted to obtain a minimum of 45 g of sorted kernels to return to the breed-

TABLE III
Effect of NIR Sorting on Purity of Waxy Kernel Samples

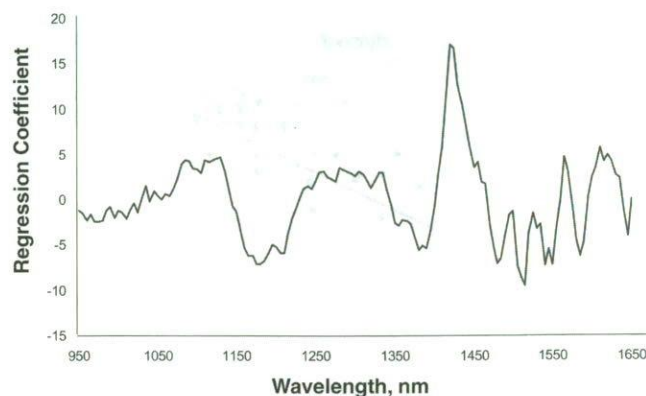| Original Waxy Kernel Frequency, %	Sorted Waxy Kernel Frequency, %	Total Original Wt, g	Wt Bin 1 (Sorted Waxy Kernels), g
18.8	75.0	586	9.30
56.3	93.8	189	79.6
64.6	93.8	306	53.2
66.7	91.7	290	117
72.9	93.8	261	118
72.9	91.7	254	104
75.0	87.5	386	45.7
77.1	89.6	190	33.5
77.1	89.6	244	59.9
77.1	97.9	626	88.7
81.3	95.8	549	211
83.3	93.8	425	85.2
83.3	93.8	519	84.1
87.5	97.9	298	62.8
Avg. 71.0	Avg. 91.8	Avg. 366	Avg. 82.3

Fig. 4. Regression coefficients (five PLS factors) of a calibration model developed to select waxy kernels based on waxy trait. Peak at 1,430 nm corresponds to the regression coefficient peak for predicting amylose content.

TABLE IV
Percentage of Waxy Wheat Kernels in Each of Four Bins After Sorting Three Replicates of Three Samples

Sample	Original Waxy Kernel Frequency, %	Bin	Sorted Waxy Kernel Frequency (%)[a] Rep 1	Rep 2	Rep 3	Average Weight Distribution (%)
1	81	1	100	100	100	14
		2	97.9	100	100	40
		3	93.8	93.8	100	14
		4	52.1	62.5	39.6	33
2	67	1	100	100	100	5
		2	93.8	100	95.8	25
		3	64.6	91.7	83.3	9
		4	31.3	29.2	2.1	61
3	56	1	100	100	100	7
		2	100	100	100	20
		3	81.3	85.4	93.8	9
		4	4.2	18.8	10.4	64

[a] No replicates for each sample and bin were significantly different ($P < 0.05$).

ing program. These samples were also sorted using the amylose calibration and compared with the results achieved with the waxy calibration. Similar results were achieved with kernels in bin 1: 97.6% pure for samples sorted using the amylose content calibration, versus 96.7% pure when using the waxy calibration. The averages were not significantly different ($P \leq 0.05$). Thus, calibrations based on amylose content or waxy trait gave similar results.

The 54-kg sample of spring waxy wheat with a 1.6% contamination of nonwaxy seed was sorted to return approximately pure waxy seed to the breeding program. For this sorting, we used the amylose calibration developed on tetraploid wheat. The sorter was adjusted to remove 10–20% of the sample in an attempt to remove all nonwaxy seed. SKNIR can process ≈1 kg/day. Thus, two sorters were run daily and often 24 hr/day to finish processing the 54-kg sample in time to meet planting deadlines. The sorting purity and rejection rate was checked daily. A baseline was collected about every 22,000 kernels, or once every several hours. Sorting resulted in a sample that when returned to the breeder had an average purity of 99.5% waxy seed (average of 51 tests, data not shown). This large quantity of seed also tested the reliability of the system because over 1 million kernels were sorted for this sample set. Both systems performed very well with little maintenance. Systems were cleaned every few days to remove excess dust.

Repeatability Tests

The three replicated field plantings of three samples that were sorted for repeatability tests showed that values between replicates were not significantly different ($P < 0.05$) (Table IV). Although there was some variation between replicates of bins 2–4, bin 1 always had 100% waxy kernels.

CONCLUSIONS

We showed that a single kernel near-infrared system could automatically select waxy wheat kernels from segregating populations or to purify an advanced breeding line. Calibrations based on amylose content or the waxy trait gave similar results. Calibrations were tested on subsequent crop years to enrich breeding lines for the waxy trait, or to purify seed lots. This technique is nondestructive, can be utilized on large seed samples, and is much faster than utilizing molecular markers or staining techniques to detect waxy kernels.

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